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Short communication

# Differentiating agents modulate topoisomerase I activity in U-937 promonocytic cells

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**Abstract**

Phorbol 12-myristate 13-acetate (PMA), *N,N′*-hexamethylenebisacetamide (HMBA) and retinoic acid induce cell differentiation in U-937 promonocytic cells. This report examines the effects of these agents on DNA topoisomerase I activity. A decrease in enzyme activity could be detected as early as 30 min after treatment with all three differentiating compounds and lasted at least 48 h. No alteration in the levels of DNA topoisomerase I transcript or protein was observed during these treatments. The results might be explained by post-translational events that render DNA topoisomerase type I less active.

*Keywords:* Topoisomerase I; Differentiation; U-937 cell

## 1. Introduction

Differentiation-inducing compounds have been considered as new agents in the treatment of some human leukemias, and their study has been facilitated by the establishment of cell lines. Human U-937 leukemia cells have been reported to differentiate into a monocytic line following exposure to phorbol 12-myristate 13-acetate (PMA) (Kolset et al., 1988), retinoic acid (Olsson and Breitman, 1982), *N,N′*-hexamethylenebisacetamide (HMBA) (Bernstein et al., 1991) and other agents.

DNA topoisomerases are enzymes that catalyze a sequential breakage and rejoining of phosphodiester bonds on DNA molecules by means of a well-characterized covalent DNA-protein intermediate. Two different types of enzymes have been identified: type I, which transiently introduce a single-strand nick in the phosphodiester backbone of the DNA, and type II, which temporarily cut both DNA strands. The biological functions of DNA topoisomerase I have not been clearly defined. However, its swivel-like activity is likely to be important in DNA replication and RNA transcription, and therefore cell differentiation (Wang, 1996). The purpose of the present study was to describe the effects of three differentiating agents, PMA, HMBA and retinoic acid, on topoisomerase I activity from U-937 cells. We demonstrate that these compounds induce a remarkable decrease in enzyme activity without affecting its mRNA and protein levels.

## 2. Materials and methods

### 2.1. Chemicals

Supercoiled pBR322 DNA was from Dupont-NEN (Wilmington, DE, USA). Fetal calf serum and cell culture media were obtained from Gibco BRL Laboratories (Gaithersburg, MD, USA). PMA, 4α-phorbol, retinoic acid, HMBA and nitroblue tetrazolium were from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

### 2.2. Cell culture

U-937 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in suspension at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin in a 5% $CO_2$ humidified incubator. Cells were routinely passaged every 3 days and seeded at a density of $2.5 \times 10^5$ cells/ml.

### 2.3. Topoisomerase I nuclear extract preparation

Crude nuclear extracts were prepared according to a previously published method (Mattern et al., 1990), with

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minor modifications. All procedures were carried out at 0–4°C. Saline-washed cells were resuspended in a small volume of hypo-osmotic buffer (5 mM potassium phosphate pH 7.0, 2 mM $CaCl_2$, 1 mM phenylmethylsulphonyl fluoride, 1 mM 2-mercaptoethanol, 0.1 mM EDTA), incubated 15 min and disrupted with 0.1% Nonidet P-40. Nuclei were collected by centrifugation, washed and resuspended in iso-osmotic buffer (5 mM potassium phosphate pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 5 mM 2-mercaptoethanol). NaCl was added to a final concentration of 0.35 M for 1 h to extract DNA topoisomerases, and samples were then centrifuged 10 min at $10\,000 \times g$. Supernatants (nuclear extract) were adjusted to 20% (v/v) glycerol and kept at −20°C until assayed. Protein determination was performed using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA).

### 2.4. Topoisomerase I activity

Enzyme activity was determined according to the procedure described previously (Crespi et al., 1988). Relaxation of supercoiled pBR322 DNA was tested in a 20-μl reaction mixture containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.2 μg plasmid DNA, and different amounts of topoisomerase I nuclear protein extract, for 30 min at 37°C. Reactions were stopped by the addition of 4 μl of stop buffer (5% sodium dodecyl sulfate (SDS), 0.25% bromophenol blue, 60% glycerol), and reaction products were analyzed by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA). DNA was visualized by staining with 0.5 μg/ml ethidium bromide. Enzyme activity was assessed by evaluating its ability to convert supercoiled circular pBR322 DNA (form I) to the relaxed state (form II). Densitometric analysis of photographed gels was performed to quantitatively determine enzyme activity. One unit of topoisomerase I activity was defined as the amount of enzyme that relaxed 50% of 0.2 μg supercoiled pBR322 DNA under the conditions described above.

### 2.5. Isolation of RNA and Northern blot analysis

Total RNA was isolated as described previously (Baldi et al., 1993). Aliquots of 15–20 μg were electrophoresed in 1% agarose-0.8 M formaldehyde gels, transferred to ZetaProbe membranes (Bio-Rad), and hybridized overnight at 45°C in a solution of 0.125 M NaCl, 0.25 M sodium phosphate pH 7.2, 5% SDS, 10% polyethylene glycol 8000, 50% formamide and $> 10^6$ cpm/ml of random primer $^{32}$P-labelled probes. Hybridization to a constitutive gene, glyceraldehyde-3-P dehydrogenase (GAPDH), was used to ensure equal RNA loading on gels. Filters were washed and exposed to Agfa Curix RP1 films. Densitometric analysis was performed to quantitate topoisomerase I expression, and results are expressed as the ratio of topoisomerase I RNA detected for each treatment relative to that of control cells, previously standardized to GAPDH mRNA levels from the same assay.

### 2.6. Western blot analysis

To examine nuclear topoisomerase I content, nuclei were prepared from control and treated cells as described above (omitting the NaCl elution step), lysed with sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol), subjected to SDS-10% polyacrylamide gel electrophoresis and transferred onto nitrocellulose. Immunostaining of immobilized proteins was carried out with a rabbit polyclonal antibody against human topoisomerase I and biotin-conjugated anti-rabbit immunoglobulin G followed by peroxidase-ABC complex (Vector Laboratories, Burlingame, CA, USA). Bands were visualized with 3,3′-

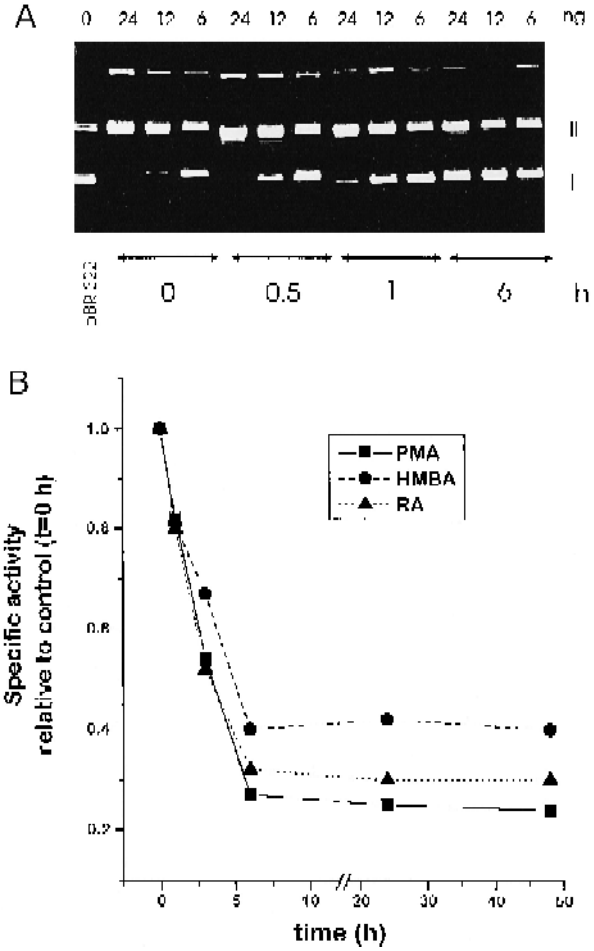


Fig. 1. Inhibition of DNA topoisomerase I activity in U-937 cells during differentiation. Cells were exposed for different periods of time to 20 nM PMA, 4 mM HMBA or 1 μM retinoic acid, and nuclear extracts were assayed for topoisomerase I activity. (A) Electrophoretic analysis of topoisomerase I activity from PMA-treated cells. Numbers at the top indicate the protein content of nuclear extracts. I: supercoiled pBR322 DNA; II: relaxed DNA. (B) Specific activity of topoisomerase I from PMA, HMBA or retinoic acid (RA)-treated cells. Results are expressed relative to those for non-treated cells. Data are representative of three independent experiments given similar results.

diaminobenzidine tetrahydrochloride/$H_2O_2$. Blots were scanned and the relative amounts of topoisomerase I were obtained by using a computer program. Values are expressed as the ratio of topoisomerase I protein detected in each experiment relative to that of untreated cells.

### 2.7. Probes and antibodies

Topoisomerase I cDNA construction (pSH5-13) and antiserum were kindly provided by Dr. L. Liu (University of Medicine and Dentistry, NJ, USA) and a 1.2-kb *Pst*I fragment of GAPDH was a generous gift from Dr. A. Kornblihtt (INGEBI-Bs.As.).

## 3. Results

The maturation of U-937 cells was assessed by determining the ability to reduce nitroblue tetrazolium, a property exhibited by differentiated cells. The percentage of differentiated cells after 3 days of treatment with 10 nM PMA, 5 mM HMBA and 1 μM retinoic acid was 78, 70 and 65%, respectively.

Since topoisomerase I activity is involved in RNA transcription and DNA replication, it was considered of further interest to explore the effects of the above-mentioned agents on this enzyme during U-937 cell differentiation.

Cells treated with PMA for different periods of times, ranging from 0.5 to 6 h, exhibited a significant time-dependent reduction in topoisomerase I activity (Fig. 1A), while 4α-phorbol, an inactive analogue of phorbol esters, was unable to modulate enzyme activity (data not shown). This effect was not limited to PMA, since HMBA and retinoic acid produced similar effects on topoisomerase I activity. These events could be detected after 30 min of treatment and were more evident after longer periods (Fig. 1B). In all cases, the progressive reduction in topoiso-

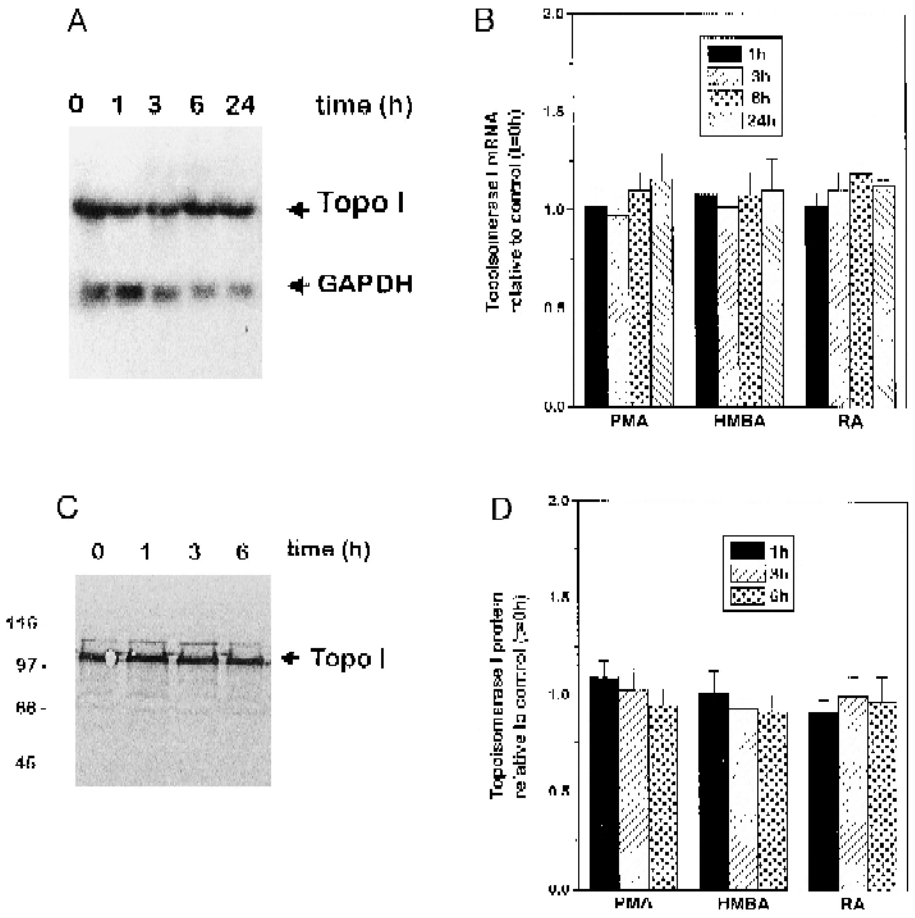


Fig. 2. Levels of topoisomerase I (Topo I) mRNA and protein in U-937 cells following treatment with differentiating agents. Cells were treated for the indicated periods of time with 20 nM PMA, 4 mM HMBA or 1 μM retinoic acid, and total RNA (20 μg) was hybridized to $^{32}$P-labeled topoisomerase I and GAPDH probes. (A) Autoradiogram of PMA-treated cells. (B) Quantitation of autoradiograms from PMA, HMBA and retinoic acid (RA)-treated cells. Values for topoisomerase I transcripts were normalized to those for control gene GAPDH and expressed relative to those for controls (non-treated cells). Values represent the mean ± S.D. ($n = 3$). Alternatively, nuclear extracts were immunoblotted and incubated with antiserum against topoisomerase I and processed as described in Section 2. (C) Western blot of nuclear extracts from PMA-treated cells. (D) Densitometric analysis of topoisomerase I protein levels from PMA, HMBA and retinoic acid (RA)-treated cells. Values are expressed relative to those for controls (non-treated cells) and represent the mean ± S.D. ($n = 3$).

merase I activity reached approximately 30% of initial values after 6 h of treatment and these levels were maintained for at least 48 h after treatment. To analyze whether the decrease in topoisomerase I activity was due to changes in the amount of enzyme present, the effects of PMA, HMBA and retinoic acid on topoisomerase I transcript and protein contents were explored. For this purpose, total RNA purified from U-937 cells was hybridized with a cDNA $^{32}$P-labeled probe complementary to topoisomerase I. Kinetic studies, extended up to 6 h in treated cells, revealed that PMA was unable to induce modifications of topoisomerase I mRNA levels (Fig. 2A). A densitometric analysis of topoisomerase I transcripts relative to those from untreated cells (Fig. 2B) showed that, as with PMA, none of the differentiating agents used in this study affected topoisomerase I expression. Fig. 2C shows a typical immunoblot analysis in which anti-topoisomerase I serum was used on proteins extracted from control U-937 cells and from cells treated with PMA for different periods of time. A densitometric representation of nuclear topoisomerase I protein levels from differentiating agent-treated cells is depicted in Fig. 2D. Values are expressed relative to the topoisomerase I protein content in non-treated cells (time = 0 h). It can be clearly seen that topoisomerase I protein levels remained constant throughout the treatment independent of the differentiating agent used.

## 4. Discussion

Much evidence supports the fact that PMA induces terminal differentiation in promonocytic U-937 cells as well as in HL-60 human promyelocytic leukemia cells. This process is associated with a loss of proliferative capacity and several phenotypic changes, including morphological reorganization, expression of specific monocyte cell markers and non-specific esterases, induction of respiratory burst (ability to reduce nitroblue tetrazolium), and differential regulation of nuclear proto-oncogenes (Collins, 1987). In this way, differentiation involves changes in the enzymatically driven DNA duplication and RNA transcription mechanisms, mechanisms in which DNA topoisomerases type I and type II play important roles.

In HL-60 cells, a number of studies link differentiation of the monocyte lineage to a decrease in DNA topoisomerase II activity, while studies on topoisomerase I still remain controversial. Our data for the human U-937 leukemia cell line clearly showed a rapid decrease in topoisomerase I activity when differentiation was induced by PMA, HMBA or retinoic acid. This down-regulation reached 30% of control values and remained constant throughout the differentiation process. Thus, the regulation of the activity of this enzyme could be considered as an early, and possibly necessary, step in the mechanism leading to differentiation of U-937 cells. The fact that camptothecin, a well-known inhibitor of topoisomerase I activity, which stabilizes the covalent DNA-enzyme intermediate, can also trigger U-937 cell differentiation (Aller et al., 1992) supports our hypothesis.

No modification in the levels of topoisomerase I mRNA or protein could be observed in U-937 cell lines committed to differentiate by PMA, HMBA or retinoic acid, suggesting the involvement of a post-translational mechanism in the regulation of this enzyme.

It is currently accepted that regulation of topoisomerase I activity by phosphorylation might be a mechanism by which protein kinases control gene expression. Topoisomerase I increases its activity after being phosphorylated in vitro on serine residues, while tyrosine phosphorylation or ADP-ribosylation decreases its activity (Wang, 1996). Protein kinase C is capable of phosphorylating topoisomerase I in vitro, restoring the DNA-relaxing activity of the in vitro-dephosphorylated enzyme in Chinese hamster cells (Pommier et al., 1990). Cardellini and Durban have reported that human topoisomerase I is rapidly phosphorylated in vivo in HL-60 cells exposed to PMA (Cardellini and Durban, 1993). Though PMA induces activation and nuclear translocation of protein kinase C in these cells, the phosphopeptide maps of topoisomerase I from PMA-treated HL-60 cells and from in vitro protein kinase C phosphorylation were not coincident. This might be due to the presence of an intermediate kinase which is under the control of protein kinase C, or to particular in vivo and in vitro conformations of the topoisomerase I protein (Cardellini and Durban, 1993). Moreover, topoisomerase I, which is also activated by casein type II kinase, shows phosphorylation sites distinct from those for protein kinase C, suggesting the presence of multiple regulatory sites modulated by different kinases. HMBA, a polar planar compound, has been described as capable of inducing differentiation of U-937 and MEL cells (erythroleukemia) via a protein kinase C-dependent pathway (Bernstein et al., 1991). Irrespective of the metabolic pathway used, our results show that the agents studied here all reduced topoisomerase I activity to the same extent, suggesting a metabolic junction in the signalling transduction pathways induced by these compounds. The present study is of special interest because it is well known that retinoic acid and HMBA affect the growth and differentiation of some cancer cells in vitro and can produce dramatic remissions in acute myelogenous leukemia and myelodysplastic syndromes (Andreeff et al., 1992; Grignani et al., 1994). In vitro studies provide a basis for the application of retinoic acid and HMBA to the clinical therapy of human cancer. The role of topoisomerase I in RNA transcription has been highlighted by its identification as the cofactor (PC3) which can both repress basal and enhance activator-dependent transcription by RNA polymerase II (Kretzschmar et al., 1993). Thus, topoisomerase I could be considered as a putative transcriptional regulating factor. Inhibition of topoisomerase I activity would result in increased torsional strain and, consequently, a progressive slowing or even

halting of transcription. However, since the inhibition of topoisomerase I activity was only partial, it might be possible that specific genes are still actively transcribed while others undergo down-regulation.

The present findings provide new insights into the mode in which differentiating agents influence cell differentiation through changes in the complex enzymatic mechanisms involved in the regulation of DNA topology and RNA transcription.

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